REMARKS

Reconsideration and allowance of the above-identified application is respectfully requested.

Claims 1-7, 9-16, 19 and 20 are pending in the application.

Claim 1 has been amended to emphasize that the raw enzyme solution is diluted in the minimal stated ratios by a factor of at least three, in effect, at least 200% with water or a buffer solution prior to treatment with the purifying agent. Support for this amendment can be found in the originally filed application including at page 4, lines 10-18 and original claims 6-8. Claims 19 and 20 have been amended to be in independent form by incorporating the subject matter of amended claim 1. No new matter has been added.

The rejection of claims 16-18 and 20 under 35 U.S.C. § 112, on page 2 of the pending Office Action, is obviated in part by the amendments to the claims set forth above. Claim 1 has been amended to better define the activity as being enzymatic and to include reference to a substrate, which provides antecedent for claim 16. Claims 17 and 18 have been cancelled to obviate the Examiner's objection.

Applicants respectfully traverse the Examiner's allegation that claim 20 is indefinite for the stated reasons. Applicants submit that the method of claim 20 is patentable because the treatment step involves use of a novel, non-obvious and commercially useful, i.e. patentable per se, enzyme solution. The claimed process step is simple and readily operable by one of ordinary skill in the art reading and comprehending the present specification.

To support Applicants' position, Applicants submit that an alternate form of claim 20 would be to have a series of individual claims based on each of method claims 1-16, each further comprising treating said substrate with said enzyme solution of enhanced activity. Present claim 20 merely simplifies the presentation of this patentable aspect.

Applicants submit that the claims fully comply with Section 112. Accordingly, withdrawal of the Section 112 rejection is respectfully requested.

In response to the Section 102 and 103 rejections, claim 1 has been amended to further define the invention to be patentably distinct over the cited seven prior art references. In particular, claim 1 has been amended to include a pre-dilution of the raw enzyme with a significant amount of aqueous solution prior to treatment with the activity agent, which is added in a minimal amount, to provide an enzyme solution of enhanced activity. None of the seven cited references singly or collectively disclose or teach all of the combined features of amended claim 1. For this reason alone, withdrawal of the Section 102 and 103 rejections of record is respectfully requested.

The rejection of claims 1-3, 5, 8, 10, 11, 14 and 19 under 35 U.S.C. § 102(b) as being anticipated by Aikat et al (biotechnology letters, vol. 23, 2001, P. 295-301) is respectfully traversed. The claimed invention is not anticipated by Aikat et al for the following reasons.

Although Aikat et al discloses use of activated charcoal in an enzyme solution, it does not disclose or teach a pre-dilution step. The Aikat et al method provides for enhancing the enzymatic activity by increasing the concentration of the desired enzyme and removal of non-essential low molecular weight proteins. This citation does not disclose or teach the invention as now claimed or provide the motivation to be combined with any of the other references for the beneficial use of a pre-dilution step according to the present invention. Accordingly, withdrawal of the Section 102 rejection is respectfully requested.

The rejection of claims 1-3, 6-8, 10 and 19 under 35 U.S.C. § 102(b) as being anticipated by Bailey (U.S. Patent No. 4,204,041) is respectfully traversed. The claimed invention is not anticipated by Bailey '041 for the following reasons.

Bailey '041 discloses use of an enzyme that is irreversibly attached to the activated carbon and wherein the latter bound material must be present in the medium where and when the enzyme-catalyzed reaction takes place. In contrast, the present invention does not involve an enzyme-bound to agent system. The reason why dilution in Bailey '041 is effected is not taught. Further, the activity of the enzyme in solution is not reported. Accordingly, there is no motivation for one of ordinary skill in the art to not

use enzyme-carbon bound systems for the purpose of enhancing activity from Bailey '041 alone or to be combined with any other reference.

Use of an enzyme attached to activated carbon is distinctly different from using an enzyme "treated" with activated carbon, and then using the resulting aqueous enzyme solution. This is further discussed in the context of Bailey '041, below. Accordingly, withdrawal of the Section 102 rejection is respectfully requested.

The rejection of claims 1-6, 8, 9 and 19 under 35 U.S.C. § 102(b) as being anticipated by Lausten et al (U.S. Publication No. 2002/0020668) is respectfully traversed. The claimed invention is not anticipated by Lausten et al for the following reasons.

Lausten et al, discloses dilution of an enzyme solution with water at an upper or maximum 1:1 ratio. The reason for this dilution is not specified and Applicants summarize it is likely to be driven by the need to control pressure and flux across membranes. No information or teachings on enzyme activity is provided. In contrast, present claim 1 defines a greater dilution ratio to provide unexpected enhanced enzyme activity. There is also no motivation disclosed in Lausten et al for one of ordinary skill in the art to pre-dilute the raw enzyme solution to at least a minimum degree and certainly not to the preferred dilution amounts as defined in the present amended claims. Accordingly, withdrawal of the Section 102 rejection is respectfully requested.

The Examiner's states on page 6 of the Office Action, that:

> "although the above references do not specifically state the enhancement of the enzyme activity, the method of treating an enzyme solution with a purifying agent, activated carbon, is the same, and further it is known in the art that activated is a purifying agent of enzymes and by purifying a substance one is further enhancing its intrinsic properties/activities, therefore, the enhancement of activity must be an inherent property of mixing such solutions together."

Applicants respectfully submit that this statement is incorrect. The Examiner contends that treatment of enzymes with activated carbon, or indeed, any purification step, should automatically lead to an enhancement of activity. However, Lausten is

silent on this matter, and Bailey '041 recorded a decrease in activity after enzymes were "mixed" with activated carbon. Others (Liljedahl, Kerkoff), hereinbelow, have discussed processes where purification led to a decrease in enzyme activity, while the Alliance Protein Laboratories site discusses the need for circular dichroism to establish equivalence of protein conformation after processing/purification, ostensibly because purification may lead to a reduction in activity. The Examiner has automatically classified Applicants' process as purification. However, as discussed in regards to Aikat et al below, Applicants' process is not simple "purification".

The rejection of claims 1, 2 and 19 under 35 U.S.C. § 102(b) as being anticipated by Bailey et al (biotechnology letters, vol. 23, 2001, P. 295-301) is respectfully traversed. The claimed invention is not anticipated by Bailey et al for the following reasons.

Bailey et al (i) does not disclose or teach a pre-dilution step, and (ii) requires an enzyme irreversibly attached to the activated carbon system that must be present in the medium where the enzyme-catalyzed reaction occurs. For the same reason presented in response to Bailey '041, hereinabove, in respect of the latter feature, this second Bailey et al reference does not disclose or teach or provide any motivation to one of ordinary skill in the art whether taken either alone or with any of the other cited references to not use an irreversibly attached enzyme-carbon entity, according to the present invention. Accordingly, withdrawal of the Section 102 rejection is respectfully requested.

The rejection of claims 12, 13 and 15-17 under 35 U.S.C. § 103(a) as being unpatentable over Shenoy et al (J. of Bioscience, vol. 7, 1985) in view of http://www.ap-lab.com/circular_dichroism.htm ("Alliance Protein Laboratories") and Lausten et al is respectfully traversed. The claimed invention is not taught or suggested by the theoretical combination of Shenoy et al, Alliance Protein Laboratories and Lausten et al for the following reasons.

Shenoy et al discuss the purification and properties of glucoamylases from different fungal sources, using circular dichroism (CD) to examine effects of pH, T,

substrate and denaturants. The binding kinetics and reaction mechanism are also discussed.

Shenoy et al notes that near-UV CD at optimal pH depends on the fungal source. A. niger and A. candidus GA showed extra peaks at 272-275 nm; troughs observed with A. niger and A. candidus were not observed with Rhizopus species. Glucoamylases from different sources had different aromatic amino acids and cysteine content. The bottom line is that the species used to produce the glucoamylase affects the near-UV CD spectrum.

The far-UV spectra also depend on species. Slight differences in magnitude and band position were observed in the 208-210nm and 217-220nm range. Some of the subtle shifts in band position are thought to be due to carbohydrate moeities in the enzyme. Enzymes from different species contain the same amount of alpha helix, but differ in beta-sheet structure.

Shenoy et al teaches that pH affects secondary structure and activity of glucoamylase. Maximum activity occurred at pH 4.8, which corresponded to the highest percentage of alpha-helix and lowest percentage of beta-sheet in all three species - A. niger, A. candidus, and Rhizopus. The lowest activity (pH 10) corresponded to higher levels of either beta-sheet or gamma (disordered structure) content. A higher pH causes ionization of tyrosine, leading to unfolding and loss of activity, consistent with involvement of ionic linkages in the native conformation.

Shenoy et al also show that increased temperature reduces helical content, ultimately leading to unfolding at higher temperatures (> 60°C). Exposure to urea reduced alpha-helix content; this paralleled a decrease in activity.

Addition of substrate also reduced the alpha-helix content, while increasing the beta-sheet content. However, near-UV bands were unaffected. Carbohydrate groups were necessary for stability, but not for activity. Perhaps the CHO moieties stabilize the enzyme against heat denaturation. Reduction of the enzyme (with and without periodate treatment) confirmed importance of the CHO for structural stability.

Essentially, Shenoy et al has used CD to show structural differences between

different sources of glucoamylase, and structural changes that occur as activity changes. In other words, following a change in activity due to a change in pH, temperature, or chemical treatment, CD can be used to elucidate the structural changes that result from such a treatment or process. One cannot, however, use a change in CD spectrum to develop a process, or to predict that a particular treatment would lead to a specific change in activity. Indeed, as noted by Alliance Protein Laboratories on their website, even though one can use CD to establish the approximate percentage of alpha-helix content, "it cannot determine which specific residues are involved in the alpha-helical portion". Applicants have also shown that the alpha-helix content of different amylases is highly dependent upon the source enzyme (Figure 3 of instant specification), but all of these enzymes are highly active, so the CD spectrum cannot be a unique predictor of activity. Thus, even though the secondary and tertiary structures of these enzymes are different, they are all active. Therefore, a specific secondary structure or a specific tertiary structure is not essential for activity, but a range of such structures may produce an "active" conformation.

Like Shenoy et al, Applicants have used CD to examine changes in structure following enzyme treatment/processing. Applicants' process has led to a change in activity, and Applicants subsequently examined the corresponding change in protein structure using CD. Applicants do not claim specific CD spectra, but rather that Applicants' process leads to a change in structure, as supported by CD spectra. Applicants' invention defines the magnitude of the spectral change that results from instant process.

With reference also again to Shenoy et al, the Examiner cited Shenoy et al as teaching that the catalytic activity of an enzyme is related to its "active" conformation, i.e. it secondary and tertiary structure. The Examiner also states that Shenoy et al teaches that the "specific activity of the purified enzymes was three times higher than that of the original non-purified glucoamylast (p400)". In fact, Shenoy et al states that "the specific activity of the purified A. candidus glucoaymlase was 436, which is three times higher than that of the parent strain already reported". The "parent strain"

referred to by Shenoy et al, is not necessarily the "original non-purified glucoamylase". The parent strain may, in fact, be a strain that has been subsequently modified or selected to produce higher glucoamylase activity – Shenoy et al is silent on this matter. However, the 3-fold improvement in activity is not automatically due to purification, as the Examiner implies. Irrespective of this, none of the CD spectra provided directly compare the purified forms developed by Shenoy et al with either their "non-purified" forms or their "parent" strains. Rather, Shenoy et al examined the structure of these purified forms using CD, and compared the spectra of these purified forms derived from different fungal species.

Alliance Protein Laboratories describes the used of CD to establish secondary and tertiary structure of proteins, and the key residues responsible for signals in the near-UV region. The authors note that CD should be used to establish conformation. The specific example cited is to "demonstrate that different lots of a protein have equivalent conformations, for example, after scale-up in the purification process". They state this in recognition of the fact that a purification process may adversely alter conformation, and thus, CD could be used to ensure that the conformation has NOT been altered by the purification process. An example is also provided where the far-UV spectrum of a recombinant protein is different from the secondary structure of the natural protein, because the recombinant protein is not properly folded (which would lead to a reduction in activity). They also note that subtle differences in structure are typically not observed in far-UV, and are often only detected in near-UV CD. The fact Applicants have observed clear differences in the far-UV spectrum is, therefore, evidence that the structural changes are not "subtle".

Alliance Protein Laboratories thus demonstrates the value of CD as a tool to examine structural changes following protein processing or changes in the protein's environment. However, CD cannot be used to anticipate or teach processes that improve activity,

Thus, based on Shenoy et al, one of ordinary skill in the art cannot predict changes in CD spectra arising from the purification process, but can appreciate the

diversity of structures arising from native (active) glucoamylases from different species, and can also see the impact of pH, temperature, urea, substrate, and chemical reduction on structure.

The Examiner cites the Alliance Protein Laboratories website in stating that "a change in the spectral range appears to be an inherent property of purification, i.e., structural change, of a protein." The Examiner goes on to state that "when purifying a protein such as enzymes, one would have a reasonable expectation of success in obtaining a CD spectrum range lower than that of the raw enzyme solution, given that purification, enhancing the catalytic activity of an enzyme, ultimately alters the secondary and tertiary structure, therefore altering the CD spectrum range. Further, it would be obvious to optimize these parameters through routine experimentation."

There are, respectfully, several inaccuracies in the preceding statement. First, the Alliance Protein Laboratories website suggests using CD to demonstrate "comparability of solution conformation after changes in manufacturing processes or formulation". Another part of their site (http://www.ap-lab.com/characterization_methods.htm) states that CD is used "for demonstrating conformational equivalence of material". These statements confirm that CD is used as a means to ensure that processing/purification has **not** altered the structure of the desired protein.

Second, there is no evidence that simple purification automatically enhances the intrinsic catalytic activity of a protein, beyond the simple removal of other materials otherwise un-needed in the enzyme-catalyzed process. In its simplest form, purification is a matter of concentration, and, thus, the activity reflects an enzyme solution that has a higher percentage of the desired protein, and a lesser percentage of the undesired materials, on a volumetric or mass basis. Such a change in concentration does not automatically imply a change in activity, or a change in structure that could go hand-in-hand with an increase (or decrease) in intrinsic activity. Indeed, activity losses are common.

For example, Liljedahl (MSc. Thesis, Uppsala University School of Engineering,

2000) states "Optimisation of a purification procedure are based on aspects such as; recovery, purity, biological activity (including unfolding, loss of co-factors, inactivation of catalytic functions, modifications of protein-protein interactions, dissociation of complexes) and time (total purification time, elution time, solubilising time, number of purification steps, etc.)". A copy of this reference has been submitted herewith.

As a further example, Kerkhoff et al. (Eur. J. Biochem. 267, 6339-6345 (2000)) aimed to purify lysophospholipid:acyl-CoA acyltransferase, and observed a loss of enzyme activity under certain purification conditions, while other purification conditions were sufficient to maintain (or recover) enzyme activity. A copy of this reference has been submitted herewith.

Consequently, contrary to the Examiner's statement, purification does not automatically imply an improvement in enzyme activity, nor does it necessarily imply an alteration in secondary and tertiary structure. In fact, changes in structure due to purification methods are often deleterious, thus leading to the desire to demonstrate "comparability of solution conformation after changes in manufacturing processes or formulation" (AP-Labs website).

Laustsen; Bailey '041: Effect of AC (Activated Carbon) on activity

There is nothing within Lausten to suggest an increase in activity arising from treatment with activated carbon; and the Examiner acknowledges that Laustsen does not "specifically state the enhancement of enzyme activity", although "the method of treating an enzyme solution with a purifying agent, activated carbon, is the same." The Examiner goes on to state that it is well known that activated carbon is a purifying agent for enzymes, and by purifying a substance, one is further enhancing its intrinsic properties/activities. The Examiner ultimately concludes that "the enhancement of activity must be an inherent property of mixing two such solutions together". This conclusion is, in fact, not justifiable.

Indeed, if this were so, Bailey '041 should have seen an increase in activity when they contacted enzyme with activated carbon during immobilization, but they did not – in fact, in their patent, they explicitly state that there was no benefit observed when some types of activated carbon were used in their process. If there was an automatic benefit associated with combining activated carbon and enzyme, as the examiner implies, then Bailey '041 should have consistently seen such a benefit, but they did not. Furthermore, their study published in Biotechnology and Bioengineering demonstrated that the intrinsic activity of the enzymes DECREASED as a consequence of contacting/attachment with activated carbon. Bailey '041 define an immobilization factor (IF) that denotes the overall change in enzyme activity during the process. The values of IF in Table I range from 0.208 to 0.381, meaning that activity was lost after contact with/immobilization to activated carbon, even on the smallest activated carbon particles. Similarly, IF values for glucose oxidase (Table III) range from 0.023 to 0.085, meaning that at least 90% of the original enzyme activity is lost after contact with activated carbon. Furthermore, Bailey '041 use their data to specifically conclude that there has been "a reduction in intrinsic activity of glucose oxidase due to immobilization" (page 1934, first three lines of paragraph 4). This is in direct conflict with the examiner's conclusion that "enhancement of activity must be an inherent property of mixing two such solutions together".

Bailey '041: Activated carbon must be continuously present

To achieve the stabilizing benefits outlined by Bailey '041, the activated carbon must remain in the system; otherwise, it cannot remove the peroxide the enzyme is continuously exposed to during the reaction catalyzed by the enzyme. This is distinct from Applicants' process, where the enzyme is contacted with activated carbon, but then the activated carbon is removed. Thus, the benefits from Applicants' process are not due to activated carbon's role as a stabilizing agent, because, in Applicants' process, only the treated enzymes are present in the reaction medium that contains the

inactivating agents described by Bailey '041. As Bailey '041 so effectively demonstrated, their process hinges on the ability of activated carbon to remove peroxide from solution; if the activated carbon is not present (or its surface is blocked, as in the case of glutaraldehyde coupling), no benefit is conferred. Since, in Applicants' process, the enzyme is separated from the activated carbon before it is used, the activated carbon is unable to provide the benefits outlined by Bailey '041. Consequently, there is nothing taught by Bailey '041 that would lead one to anticipate that treatment of an enzyme by an appropriate amount of activated carbon could lead to an increase in the activity of the enzyme or a change in the structure of the enzyme, particularly if the enzyme is ultimately used independently of the activated carbon itself.

Aikat: Distinction from Simple Purification

The key to the results of Aikat et al. is their electrophoresis results. The images of the crude protein and the charcoal-treated proteins show that the smaller proteins were almost completely removed by the activated carbon. This is clearly a quintessential case of protein purification.

By comparison, the claimed process results in an extra band when glucoamylase is treated, and the smaller proteins are more abundant when amylase is treated (shown as A, A', B, B' in figure 3). The observations are consistent with a structural change in the native protein, but <u>not</u> consistent with the simple purification described by Aikat. It can therefore be concluded that Aikat et al. have performed a simple purification – small proteins, debris, and extracellular nucleic acids have been removed by adsorption and/or size exclusion, while leaving the treated protein unaffected. In contrast, the claimed process leads to transformation of the enzyme.

Unexpected Results

To summarize, Applicants respectfully submit that there is nothing in any of the cited references that could possibly teach the surprising experimental results seen in Figures 2 and 3 of instant specification that following significant dilution and subsequent treatment with activated charcoal, results in at least the original level of enzyme activity, and by so doing, provides a significantly more efficacious use of the enzyme and provides significant cost benefits.

In view of the many differences between the claimed invention and the theoretical combination of cited references, and the unexpected advantages of the claimed invention, withdrawal of the Section 103 rejection is respectfully requested.

In view of all of the objections and rejections of record having been addressed, Applicants submit that the present application is in condition for allowance and Notice to that effect is respectfully, requested.

Respectfully submitted,
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